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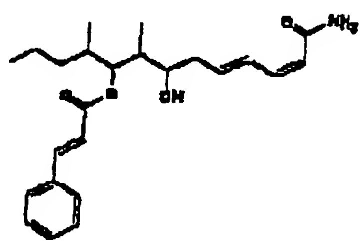
(54) Name of invention An anti-fungal biotic and its manufacturing process

(57) Summary
[Structure] The anti-fungal biotic, YL-03709B-A, has a structure as shown below (I)

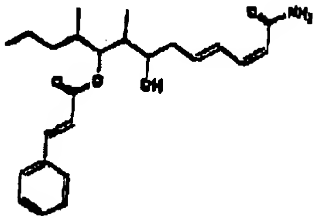
[Illustration of chemical structure I]



(I)

[Efficacy] Its strong anti-fungal efficacies are useful as an anti-fungal drug

[Patent application range]
[Claim 1] Anti-fungal biotic with the chemical structure as shown below (I).



(I)

[Claim 2] A process to manufacture the anti-fungal biotic YL-03709B-A. It consists of cultivating a Bacillus microbe which has abilities to produce YL-03709B-A, and extracting the substance from the culture medium.

[Claim 3] The bacillus microbe is Bacillus sp. YL-03709B (FERM P-14126) as used in the above Claim 2.

[Detail of invention]
[0001]
[Application area] This invention is related to medicine, specifically an anti-fungal biotic, and its manufacturing process by fermentation

[0002]
[Conventional technologies] Since 1950s the industry has witnessed remarkable development and applications of anti-biotics, leading to almost complete eradication of conventional infectious diseases. On the other hand, the recent years saw a different kind of diseases (opportunistic infectious diseases). They are caused by weak pathogens as traditionally known. They occur

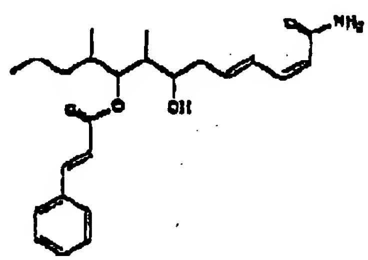
(1) when the patient's immunity decreases from , e.g., immuno-insufficiency, malignant tumors and/or use of drugs such as immuno-suppressers and anti-inflammatory agents
(2) Suppressing co-inhabiting microbes by the use of antibiotics
(3) Infection arising in hospitals

These so-called opportunistic infections are commonly caused by fungi. Conventionally drugs such as Amphoterin B, Griseofulvin and Nystatin are used for the treatment.

[0003]
[Objectives of our invention]
As above unconventional infectious diseases by fungi have been on the rise in the recent years, and effective anti-fungal drugs have been much anticipated. Through our research into naturally produced substances using microbes, a compound was identified which has strong efficacy in controlling undesirable fungi. The compound is produced by a microbe from Bacillus group. The microbe produces the compound in the culture medium, and the compound is isolated from the medium. Thereby, the objective of this invention is to offer a novel anti-biotic which has strong anti-fungal capabilities. Another objective is to offer a process to manufacture the above compound. Also, this invention is to offer a microbe which can produce such a compound.

[0004]
[Method to attain the objectives]
The anti-biotic compound is coded as YL-03709B-A and its chemical structure is shown below (I).

[0005]
[Illustration of chemical structure 2]



(I)

[0006] It is a manufacturing process by culturing a microbe which belongs to Bacillus group. The microbe produces the compound in the culture medium. Subsequently, the accumulated compound is isolated from the medium.

[0007] This noval anti-fungal agent is obtained from a microbe which can produce Compound YL-03709B-A. Thus manufactured product has asymmetric carbon atoms in the structure, therefore, a number of isomers are possible. The compound may be, thereby, mixtures of different isomers. The microbial strain which can produce the YL-03709B-A compound is Bacillus sp. as isolated from soils which were collected on Ishigaki Island, Okinawa. The characteristics are as below.

[0008] The characteristics of Bacillus sp. YL-03709B strain

1. Morphological description

When cultured on a agar medium for 5 days at 32 °C, the cells are 0.6~1. 0 x 2.0~7.0 μm in size and rod shaped. It is gram positive. It has mobilities. A spore is produced around the centre to toward an end of the cell. The spore is oval in shape. It is resistant to heat at 100 °C for 10 minutes.

[0009] Growth in various culture media

End of page 2

The table shows the growth of the microbe on various media. The culture is 2 ~ 7 days at 32 °C. The observation is according to the standard method. The description of shade is according to the Color Standard (Japan Color Shade Institute).

(1) On meat extracts agar medium

Glossy and non-transparent colonies with shade ranging from creamy color to yellow/brown/grey shades. No pigments are produced.

(2) On glucose/meat extracts agar medium

Same as above

(3) Meat broth liquid medium

It forms surface film, and the medium's upper layers become cloudy.

[0010] 3. Physiological description
It is tabulated below (Table 1).

Reduction of nitric salts	Positive
VP test	Negative
Production of indol	Negative
Production of hydrogen sulfide	Negative
Hydrolysis of starch	Negative
Use of citric acid	Positive
Use of maronic salts	Positive
Liquefaction of gelatin	Positive
Hydrolysis of lysine	Negative
Hydrolysis of ornithin	Negative
Hydrolyses of arginine	Negative
Use of aesculin	Positive
Crease	Negative
Decomposition of tyrosine	Negative
Production of dioxyacetone	Negative
Oxydase	Positive
Catalase	Positive
Growth temperature	15 ~ 40 ºC
Optimal growth temperature	24 ~ 37 ºC
Growth pH	6 ~ 9
Optimal growth pH	7 ~ 8
Growth under anaerobic conditions	Negative
OF test	No decomposition
Growth in meat extracts medium with NaCl	Positive at <9% NaCl

[0011] Use of carbon sources
Table 2 shows the results.

Glucose	+	Sucrose	-
Xylose	-	Rhamnose	-
Arabinose	-	Mannitol	+
Mannose	+	Inositol	-
Fructose	+	Adonitol	-
Maltose	+	Sorbitol	-

note: +: positive growth -: no growth

[0012] 5. Analysis of menaquinone
The Primary menaquinone is MK-7

[0013]
Based on the above characteristics, the strain is gram positive and has mobilities. It is aerobic, VP test negative, oxydase test positive, catalase test positive. It has abilities to reduce nitrate salts. It grows in moderate temperature ranges of 15 – 40 °C. We conclude that the microbe belongs to Bacillus group (ref. Bergey's Manual of Systematic Bacteriology,1984, and other sources). It is similar to other Bacillus organisms such as B. badius, B. brevis, and B. circulans. Comparison with these organisms are listed in Table 3.

[0014]
[Table 3]

<u>End of page 3</u>

Size	Our Strain 0.4–1.0 x 2–7 μm	B. badius, 0.8–1.2 x 1.5–4 μm	B. brevis, 0.6–0.9 x 1.5–4 μm	B. circulans 0.5–0.7 x 2–5 μm
Oxydase	+	ND	ND	-
Catalase	+	+	+	+
Anaerobic growth	-	-	-	d
VP test	-	-	-	-
Gelatin liquefaction	+	ND	d	d
Starch hydrolysis	-	-	d	+
Use of citric acid	+	-	d	d
Tyrosine decomposition	-	-	ND	ND
Reduction of nitrates	+	-	d	d
Production of indol	+	+	+	+
Production of dioxyacetone	-	-	-	-
NaCl in medium				
2% NaCl	+	ND	ND	ND
5% NaCl	-	+	-	d
Temperature				
5 °C	-	-	-	-
10 °C	-	-	-	d
30 °C	+	+	d	+
40 °C				
50 °C	-	+	d	-
55 °C	ND	-	d	-
60 °C	ND	-	-	-

note: ND: not determined d: 11–89% of strains positive

[redacted]

[0015] According to Bergey's Manual of Systematic Bacteriology,1984, The common characteristics are gram positive, a spore is formed in each of the cell at the centre to toward an end. They are negative in the VP test, indol reaction, and dioxyacetone test. In contract, our strain is different in size as compared to the other three microbes. Also, B. badius can grow on 5% NaCl meat extracts medium, and also at temperatures as high as 50 ºC. Other notable difference is that B. circulans can hydrolyze starch. As such, our strain is not the same as the other three microbes.

[0016].
Therefore our strain is named as Bacillus sp. YL-03709B. Cultures of this strain is forwarded to the Industrial Technology Institute Bio-Science Laboratory. Its I.D. is FERM P-14126. The microbe is subject to mutation. Therefore, mutant strains as obtained by ultraviolet and X-rays irradiation as well as by chemical treatment are also part of this invention.

[0017] [Manufacturing process]
Bacillus sp. YL-03709B is cultured in a medium under aerobic conditions. The medium could be any such as synthetic or natural medium where the microbe can grow. The nutrients required are those commonly available. Examples are peptone, meat extracts, Corn???(translator note: unable to find the corresponding English spelling), cotton seeds flour, peanut flour, yeast extracts, wheat germs, casein hydrate, fish meal, inorganic or organic nitrogen sources such as sodium and ammonium nitrates. Carbon sources may be molasses, glucose, mannose, fructose, mannitol, glycerin, potato starch, corn starch, dextrin, soluble startch and fats.

[0018] Other required additives include sulfate, chloride, phosphate or carbonate forms of Na, K, Mg, Ca, Zn, Fe, Co, Cu. Further amino acids such as valine, leucine, iso-leucine, phenylalanine, tryptophan, methionine, lysine, arginine, glutamic acid, asparaginic acid, vitamines, methyl-oleine, lard, silicone oils, surfactants and anti-form agents. Other additives may also be used to support the growth of the YL-03709B microbe.

[0019] Culture is either in liquid or on solid medium. Either still, stir or shaker culture is possible for liquid cultivation. Good aeration is desirable. The useful temperature range is within 15 – 40 ºC, and the 24– 37 ºC range is more favorable. The pH of the medium is 6–9, and the 7–8 range is more favorable. Culture time ranges from 10 to 168 hrs, whereas the best efficacies are obtained in 24–120 hrs.

[0020] The commonly used extraction and purification methods are sufficient to isolate the target product. First the culture is subject to separation using centrifuge or filtration with filter aids. The filtrate is then extracted using solvents such as ethylacetate and chloroform. Or, it may be isolated first adsorbing on adsorbents and subsequently eluting it using solvents. The adsorbents may be porous materials such as AmberliteXAD-2, DiaionHP-20, DiaionCHP-20 and DiaionSP-900.

[0021] Elution can be accomplished using admixtures of water and organic solvents such as methanol, ethanol, acetone and acetonitrile. The concentration of the organic solvent progressively increases as the number of elution stage increases. When direct extraction using solvents such as ethylacetate and chloroform is used, the solvent is mixed with the culture medium filtrate, the mixture is shaken and extracted into the solvent phase. Following extraction, the antibiotic fraction is dried through column chromatography with silica gel or ODS, etc. Centrifugal liquid-liquid partition chromatography or HPLC with ODS is required to further purify the product.

[0022]
[Example] An example is described below. However, our invention is not limited within the example.

[0023] (An example) The culture medium consists of 1% glucose, 2% potato starch, 0.5% meat extracts, 0.5% polypeptone and 0.4% calcium carbonate. The medium pH is 7.0. The medium is transferred into conical flasks (100 ml/flask), and subsequently sterilized at 121 °C for 20 minutes. Then, they are inoculated to grow inoculant. The microbe has been cultured on the Bennett agar medium. It is stir cultured at 28 °C for 72 hrs under an aerobic condition. For anti-biotic production the same medium is inoculated using the above inoculant (2%). Again, it is stir cultured at 28 °C for 72 hrs under an aerobic condition.

[0024] Thus obtained culture (2.5 L) is filtered. The filtrate is adjusted to pH 3, subsequently extracted with ethylacetate. The extract is concentrated under vacuum pressure to yield 344 mg material. It is further subject to flash chromatography (Merk ??gel 60). The chloroform/methanol (9:1) fraction is further fractionated into two phases using ethylacetate and saturated NaHCO3 water solution. An yield of 42 mg results in the ethylacetate fraction. It is further subject to flash chromatography (YMC-GEL). Subsequently the methanol/water (9:1) eluate is purified using HPLC (Pagasil ODS, acetonitrile/water=5:46). The yield of the purified product is 4.9 mg.

[0025] Thus obtained YL-03709B has the following physico-chemical properties.

(1) Color and property: colorless, transparent or semi-transparent, amorphous, viscous material

(2) Acid, neutral or base: neutral

(3) Solubility: Soluble in methanol, ethanol, acetone, ethylacetate, chloroform, dimethylsulfoxide. Partially soluble in water. Not soluble in hexane

(4) Molecular weight: 399

(5) Chemical formula: $C_{24}H_{33}NO_4$

(6) Relative angular rotation: $[\alpha]_D$ - 106.1 (c 0.33, methanol)

(7) Mass spectrum (FAB-Mass): m/z 400.244 9(MH+: $C_{24}H_{34}NO_4$, Δ-3.8 mmu

(8) Ultraviolet light spectrum in methanol: λ_{max} 217 (ε19000), 222 (ε18000), 263 (ε31000) nm

(9) Infrared absorption spectrum (Film Method): 3340, 2960, 1670 cm^{-1}

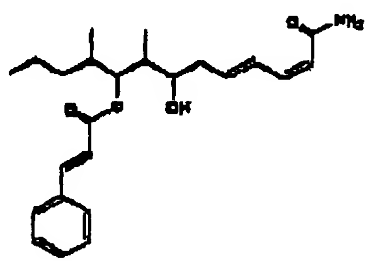
(10) ^{1}H-NMR spectrum (in $CDCl_3$, 500MHz): as shown in Figure 1

(11) ^{13}C-NMR spectrum (in $CDCl_3$, 125MHz): as shown in Figure 2

As above the chemical structure of YL-03709B-A is identified as below.

[0026]

[Chemical structure illustration 3]



(I)

[0027]

[Efficacy] The antibiotic YL-03709B-A demonstrates strong anti-fungal activities, therefore, it can be used as medicine, specifically as an anti-fungal agent. Figure 4 lists such activities. The evaluation (MIC, μg/ml) is conducted on the Sabouraud/dextrose agar medium.

[0028]

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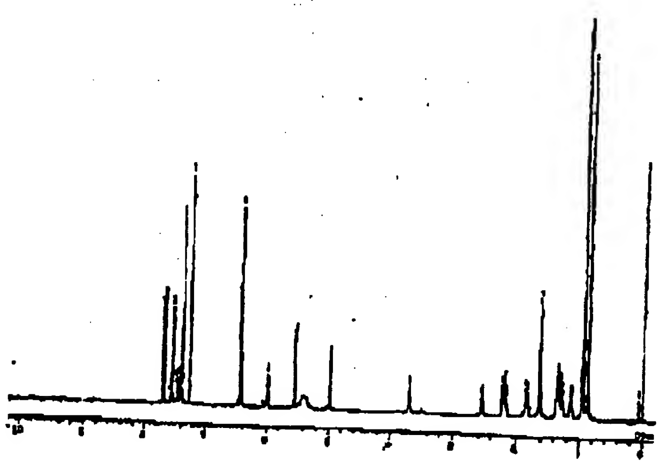
Table 4
Anti-fungal activities of antibiotic YL-03709B-A

Test Microbes	MIC , μg/ml)	
Candida albicans	25	
Candida parapellosis	25	
Plebia angusia *	0.75	(* not legible)
Rhodotorula acuta	0.05	
Trigonopsis variabillis	0.25	
Sacchromyces cerevisiae	50	
Sacchromyces sake	50	
Cryptorrcus sp.	0.25	
Aspergillus niger	>/=50	

Figure 1: ^{1}H-NMR spectrum of YL-03709B-A



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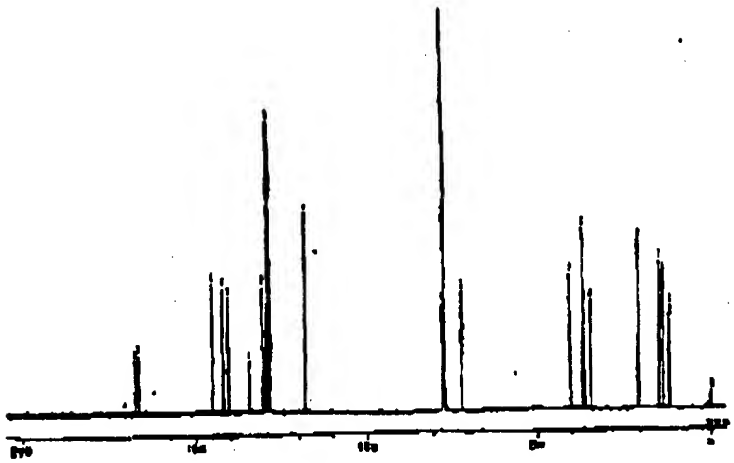
[redacted]

Figure 2: ^{13}C-NMR spectrum of YL-03709B-A



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